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| (21) International Application Number: PCT/DK91/00243 (22) International Filing Date: 23 August 1991 (23.08.91) (30) Priority data: 2042/90 24 August 1990 (24.08.90) DK (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK). (72) Inventors; and (75) Inventors/Applicants (for US only) : MIKKELSEN, Jan, Møller [DK/DK]; Snerlevej 20, DK-2820 Gentofte (DK). HANSEN, Lone, Kierstein [DK/DK]; Ulrikkenborg Allé 55, DK-2800 Lyngby (DK). (74) Common Representative: NOVO NORDISK A/S; Patent Department, Novo Allé, DK-2880 Bagsvaerd (DK). | | (81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US. Published <i>With international search report.</i> |
| (54) Title: ENZYMATIC DETERGENT COMPOSITION AND METHOD FOR ENZYME STABILIZATION (57) Abstract The invention relates to a detergent composition comprising a protease and one or more other enzymes, as well as comprising a reversible protease inhibitor of the peptide or protein type. | | |

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ENZYMATIC DETERGENT COMPOSITION AND METHOD FOR ENZYME STABILIZATION

TECHNICAL FIELD

The present invention relates to a detergent composition comprising
5 a protease and a second enzyme (which may be another protease or a non-
proteolytic enzyme), to a method for stabilizing an enzyme in the presence of a
protease and to an enzymatic detergent additive comprising a protease and a
second enzyme.

BACKGROUND ART

10 Proteases are widely used as ingredients in commercial detergents,
including liquids. Two different proteases may be used together (US 4,511,490,
WO 88/03946). Other enzyme types (i.e. non-proteolytic) may also be used in
detergents, e.g. amylase, cellulase, lipase or peroxidase.

A major problem in formulating enzymatic detergents, especially
15 liquid detergents, is that of ensuring enzyme stability during storage. For a
detergent containing a protease together with another enzyme, the stability
problem is aggravated as the protease is liable to digest and deactivate the other
enzyme (i.e. the other protease or the non-proteolytic enzyme).

WO 89/04361 discloses a detergent containing a protease and a
20 lipase, where improved lipase stability is achieved by selecting a specified groups
of lipases and a specified group of proteases.

STATEMENT OF THE INVENTION

We have found that, surprisingly, the stability of an enzyme in a
detergent containing a protease can be improved by incorporation of a reversible
25 protease inhibitor of the peptide or protein type.

Accordingly, the invention provides a detergent composition comprising a protease and one or more other enzymes, characterized by further comprising a reversible protease inhibitor of the peptide or protein type. In another aspect, the invention provides a method for stabilizing an enzyme in the presence of a protease, characterized by incorporating a protease inhibitor. A further aspect of the invention provides an enzymatic detergent additive comprising a protease and one or more other enzymes in the form of a stabilized liquid or a non-dusting granulate, characterized by further comprising a reversible protease inhibitor of the peptide- or protein-type.

10 JP-A 62-269689 discloses improvement of the stability of a protease in a liquid detergent by incorporation of a protease inhibitor, but no other enzymes were present.

DETAILED DESCRIPTION OF THE INVENTION

Protease

15 The protease used in the invention is preferably of microbial origin. It may be a serine protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, e.g. subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (both described in WO 89/06279) and mutant
20 subtilins such as those described in WO 89/06279. Examples of commercial *Bacillus* subtilisins are Alcalase®, Savinase® and Esperase®, products of Novo Nordisk A/S. Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270.

The amount of protease in the detergent will typically be 0.2-40 μ M,
25 especially 1-20 μ M (generally 5-1000 mg/l, especially 20-500 mg/l).

Other enzymes

The other enzyme(s) used in the invention may be another protease (e.g. of the type described above) or a non-proteolytic enzyme, e.g. an amylase, a cellulase, a lipase or an oxidoreductase, such as a peroxidase. The non-
5 proteolytic enzyme is preferably of microbial origin, e.g. derived from a strain of *Bacillus*, *Humicola*, *Pseudomonas*, *Coprinus* or *Fusarium*.

The amount of the other enzyme(s) in the detergent will typically be 0.2-40 μ M, especially 1-20 μ M (generally 5-1000 mg/l, especially 20-500 mg/l).

Inhibitor

10 The inhibitor used in the invention may be any inhibitor of the peptide or protein type that reversibly inhibits the protease in question, e.g. those described in Lakowski, Jr. & Kato, *Ann.Rev.Biochem.* (1980) 49:593-626 and S. Murao et al., in *Protein Protease Inhibitor - The Case of Streptomyces subtilisin Inhibitor* (1985) at pp. 1-14. Examples are trypsin inhibitors of Family IV
15 (described in the cited references) and subtilisin inhibitors of family III, VI and VII. More particular examples are *Streptomyces subtilisin inhibitor* (SSI); plasminostreptin from *Streptomyces antifibrinolyticus*; barley subtilisin inhibitor CI-1 (e.g. described in Williamson et al., *Plant Mol. Biol.* 10, 1988, pp. 521-535) and CI-2 (e.g. described in Williamson et al., *Eur. J. Biochem.* 165, 1987, pp. 99-106);
20 potato subtilisin inhibitor I (e.g. described in Cleveland et al., *Plant Mol. Biol.* 8, 1988, pp. 199-207); tomato subtilisin inhibitor (e.g. described in Graham et al., *J. Biol. Chem.* 260, 1985, pp. 6555-6560); eglin C from leech (e.g. described in Seemüller et al., *Hoppe-Seylers Z. Physiol. Chem.* 361, 1980, pp. 1841-1846); *Vicia faba* subtilisin inhibitor (e.g. described in Svendsen et al., *Carlsberg Res. Commun.* 49, 1984, pp. 493-502); and leupeptin inhibitor (e.g. described in S.
25 Kondo et al., *J. Antibiot.* 22, 1969, pp. 558-568).

Furthermore, the inhibitor may be a modified subtilisin inhibitor of Family VI with a weaker binding affinity for the protease. Such a modified inhibitor may have one or more of the following amino acid substitutions at the indicated
30 positions (numbered from the reactive site of the inhibitor, P1, P2 etc. are in the

direction of the N-terminal and P'1, P'2 etc. are in the direction of the C-terminal of the inhibitor molecule):

P4: Val, Pro, Trp, Ser, Glu or Arg

P3: Tyr, Glu, Ala, Arg, Pro, Ser, Lys or Trp

5 P2: Ser, Lys, Arg, Pro, Glu, Val, Tyr, Trp or Ala

P1: Arg, Tyr, Pro, Trp, Glu, Val, Ser, Lys or Ala

P'1: Gln, Ser, Thr, Ile or Pro,

P'2: Val, Glu, Arg, Pro or Trp,

P'3: Glu, Gln, Asn, Val, Phe or Tyr.

10 A preferred modified inhibitor is Cl-2 substituted with Arg, Pro or Glu at position P3, Lys or Arg at P2, and/or Glu, Arg or Pro at P1.

Modified inhibitors may be produced by known recombinant DNA techniques. Briefly, a DNA sequence (cDNA or a synthetic gene) encoding a known inhibitor is subjected to mutagenesis in order to replace the codon(s) for
15 the amino acid(s) to be substituted with a new codon (codons) for the desired amino acid substitution(s). This may preferably be carried out by oligonucleotide-directed site-specific mutagenesis in bacteriophage M13 vectors (e.g. M.J. Zoller and M. Smith, Meth. Enzymol. 100 (1983) 468-500), in double-stranded DNA vectors (e.g. Y. Morinaga et al., Biotechnology (July 1984) 636-639), or by the
20 polymerase chain reaction (PCR) (e.g. R. Higuchi, Nucl. Acids. Res. 16 (1988) 7351-7367).

The mutant gene is subsequently expressed in a suitable host strain. Suitable hosts are bacteria (e.g. strains of *Escherichia coli* or *Bacillus*), fungi (e.g. strains of *Saccharomyces cerevisiae* or filamentous fungi like *Aspergillus*), plants
25 such as tomato or potato or established human or animal cell lines. To accomplish expression, the mutant gene has to be inserted in an expression plasmid with promoter and terminator DNA elements for the formation of translatable mutant inhibitor mRNA in vivo. The plasmid is introduced into the host by genetic transformation. The choice of expression plasmid is dependent
30 on the type of host strain used. The expression of the mutant inhibitor may be

done intracellularly or extracellularly. In the latter case, the DNA sequence coding for the mutant inhibitor is fused in frame to a DNA sequence encoding a suitable peptide signalling secretion. The secretion signal should preferably be cleaved off in vivo, resulting in secretion of the mature mutant inhibitor into the growth medium.

The amount of inhibitor preferably corresponds to a molar ratio of inhibitor reactive site to protease active site above 0.6, more preferably above 0.8 and most preferably above 1. The ratio is generally below 10, usually below 5.

The type and amount of inhibitor is preferably chosen so as to provide at least 60% (e.g. at least 80%) inhibition in the detergent as such and below 10% inhibition when the detergent is diluted with water for use in washing, typically at a concentration of 0.3-10 g/l.

Detergent

The detergent of the invention may be in any convenient form, e.g. powder, granules or liquid. The invention is particularly applicable to the formulation of liquid detergents where enzyme stability problems are pronounced. A liquid detergent may be aqueous, typically containing 20-70% water and 0-20% organic solvent (hereinafter, percentages by weight).

The detergent comprises surfactant which may be anionic, non-ionic, cationic, amphoteric or a mixture of these types. The detergent will usually contain 5-30% anionic surfactant such as linear alkyl benzene sulphonate (LAS), alpha-olefin sulphonate (AOS), alcohol ethoxy sulphate (AES) or soap. It may also contain 3-20% anionic surfactant such as nonyl phenol ethoxylate or alcohol ethoxylate.

The pH (measured in aqueous detergent solution) will usually be neutral or alkaline, e.g. 7-10. The detergent may contain 1-40% of a detergent builder such as zeolite, phosphate, phosphonate, citrate, NTA, EDTA or DTPA, or it may be unbuilt (i.e. essentially free of a detergent builder). It may also contain other conventional detergent ingredients, e.g. fabric conditioners, foam boosters, bactericides, optical brighteners and perfumes.

Detergent additive

The protease, other enzyme(s) and inhibitor may be included in the detergent of the invention by separate addition or by adding the combined additive provided by the invention. The additive will usually contain 0.2-8 mM 5 protease (0.5-20%) and 0.2-8 mM (0.5-20%) of the second enzyme, and have an inhibitor/protease ratio as described above.

The detergent additive may be in liquid form for incorporation in a liquid detergent. A liquid additive may contain 20-90% propylene glycol; 0.5-3% (as Ca) of a soluble calcium salt; 0-10% glycerol; minor amounts of short-chain 10 fatty acids and carbohydrate; and water up to 100%.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is illustrated in further detail in the following examples with reference to the appended drawings, wherein

Fig. 1 is a graph showing the residual activity (in %) after 13 days at 15 room temperature of lipase in a detergent composition containing lipase and protease alone compared to a composition containing lipase, protease and Streptomyces subtilisin inhibitor;

Fig. 2 is a graph showing the residual activity (in %) after 13 days at room temperature of lipase in a detergent composition containing lipase and 20 protease alone compared to a composition containing lipase, protease and barley subtilisin inhibitor CI-2;

Fig. 3 is a graph showing the residual activity (in %) after 43 hours at room temperature of lipase in the presence of protease with or without added leupeptin inhibitor; and

Fig. 4 is a graph showing the residual activity (in %) after 10 days at room temperature of cellulase in the presence of protease with or without added Streptomyces subtilisin inhibitor; and

Fig. 5 is a graph showing the residual activity (in %) after 10 days at room temperature of cellulase in the presence of protease with or without added CI-2 inhibitor.

EXAMPLE 1

A concentrated liquid detergent was formulated as follows (% by weight of active substance):

| | | | |
|----|------------------------------|-------|-------|
| 10 | LAS (Nansa 1169/p) | 5% | |
| | AES (Berol 452) | 5 | |
| | Oleic:coco fatty acid (1:1) | 10 | |
| | AE (Dobanol 25-7) | 15 | |
| | Triethanolamine | 5 | |
| 15 | NaOH | | 1.1 |
| | SXS | 3 | |
| | Ethanol | 4.8 | |
| | Propylene glycol | 8 | |
| | Glycerol | 2 | |
| 20 | CaCl ₂ | | 0.045 |
| | Sodium citrate | 0.089 | |
| | Phosphonate (Dequest 2060 S) | 0.5 | |
| | pH | 8.0 | |

A detergent according to the invention was prepared by addition of Streptomyces subtilisin inhibitor (SSI, 0.05 mg/ml, 4.5 μ M) to a detergent of the composition: 52 (v/v) % of the above concentrated detergent in water containing

10 mg/ml (300 μ M) *Humicola* lipase (LipolaseTM) and 0.1 mg/ml (3.6 μ M) Savinase .

Another detergent was prepared by addition of inhibitor CI-2 (0.03 mg/ml, 3.3 μ M) to a detergent of the composition 55 (v/v) % concentrated
5 detergent in water containing 10 mg/ml (300 μ M) *Humicola* lipase (LipolaseTM) and 0.1 mg/ml (3.6 μ M) Savinase®.

Both detergents were stored for 13 days at room temperature together with reference detergents of the same composition, but without inhibitor. The lipase activity was measured at various times and expressed in % of initial
10 lipase activity. The results, shown in the two figures, demonstrate a pronounced stabilizing effect on the lipase by addition of the protease inhibitor.

EXAMPLE 2

The protection of lipase from proteolytic degradation in the presence of a protease inhibitor was determined by adding 0.67 g/l leupeptin inhibitor to a
15 mixture of 0.5 g/l *Pseudomonas cepacia* lipase and 2 g/l *Fusarium* protease in 50 mM Tris-HCl, pH 8.0, at 20°C and measuring the residual lipase activity (in %) after 43 hours. From the results shown in Fig. 3 it appears that there is very little degradation of the lipase in the presence of the leupeptin inhibitor, whereas the lipase is almost completely degraded when no inhibitor is added. The protease
20 activity may be restored by dilution. After storage for 43 hours followed by 100-fold dilution, the protease activity was 327 U/ml (U = arbitrary units established by means of the synthetic substrate N-p-tosyl-Gly-Pro-Arg-p-nitroanilide) in the preparation containing lipase and protease, and 366 U/ml in the equivalent preparation which also contains the leupeptin inhibitor.

EXAMPLE 3

A concentrated liquid detergent was formulated as follows (% by weight of active substance):

| | | |
|---|--------------------|-----|
| | LAS (Nansa 1169 P) | 10% |
| 5 | AEO (Berol 160) | 15% |
| | Ethanol | 10% |
| | Triethanolamine | 5% |

A detergent according to the invention was prepared by addition of *Streptomyces subtilisin* inhibitor (SSI, 0.09 mg/ml, 7.7 μ M) to a detergent (90% (w/w) of the above concentrated detergent in water) containing 0.12 mg/ml (3.3 μ M) *Humicola* cellulase and 0.18 mg/ml (6.7 μ M) Savinase®.

Another detergent was prepared by addition of inhibitor CI-2 (0.07 mg/ml, 7.8 μ M) to a detergent (90% (w/w) of the above concentrated detergent in water) containing 0.12 mg/ml (3.3 μ M) *Humicola* cellulase and 0.18 mg/ml (6.7 μ M) Savinase®.

Both detergents were stored for 10 days at room temperature together with a reference detergent without any inhibitor. The residual cellulase activity was measured at various times and expressed in % of initial cellulase activity. The results, shown in Fig. 4 and 5 demonstrate a pronounced stabilizing effect on the cellulase by addition of protease inhibitor, especially with SSI.

CLAIMS

1. A detergent composition comprising a protease and one or more other enzymes, characterized by further comprising a reversible protease inhibitor of the peptide or protein type.
- 5 2. A composition according to the preceding claim, wherein the molar ratio of inhibitor reactive site to protease active site is above 0.6, preferably 1-10.
3. A composition according to either preceding claim, wherein the amount of protease is 0.2-40 μM , preferably 1-20 μM .
4. A composition according to Claim 1, wherein the protease is a
10 serine protease, preferably an alkaline microbial protease or a trypsin-like protease.
5. A composition according to Claim 4, wherein the alkaline microbial protease is a subtilisin, preferably derived from *Bacillus*, most preferably subtilisin Novo, subtilisin Carlsberg, BPN', subtilisin 309, subtilisin 147 or subtilisin 168.
- 15 6. A composition according to Claim 4, wherein the trypsin-like protease is trypsin or is derived from *Fusarium*.
7. A composition according to any preceding claim, wherein the inhibitor is a trypsin inhibitor of family IV or a subtilisin inhibitor of family III, VI or VII.
- 20 8. A composition according to any preceding claim, wherein the other enzyme is also a protease, preferably of the type defined in any of Claims 4 - 6.

9. A composition according to any of Claims 1 - 7, wherein the other enzyme is a non-proteolytic enzyme, preferably an amylase, a cellulase, a lipase or an oxidoreductase, such as a peroxidase.
10. A composition according to the preceding claim, wherein the
5 enzyme is of microbial origin, preferably derived from *Bacillus*, *Humicola*, *Pseudomonas*, *Coprinus* or *Fusarium*.
11. A composition according to any preceding claim, wherein the degree of protease inhibition in the detergent is at least 60%.
12. A composition according to any preceding claim, wherein the
10 degree of protease inhibition in a 1% detergent solution in water is below 10%.
13. An aqueous liquid detergent composition according to any preceding claim.
14. A method for stabilizing an enzyme in the presence of a protease, characterized by incorporating a protease inhibitor of the peptide or protein type.
- 15 15. A method according to Claim 14 for stabilizing an enzyme in a detergent, preferably an aqueous liquid detergent.
16. An enzymatic detergent additive comprising a protease and one or more other enzymes in the form of a stabilized liquid or a non-dusting granulate, characterized by further comprising a reversible protease inhibitor of the peptide
20 or protein type.

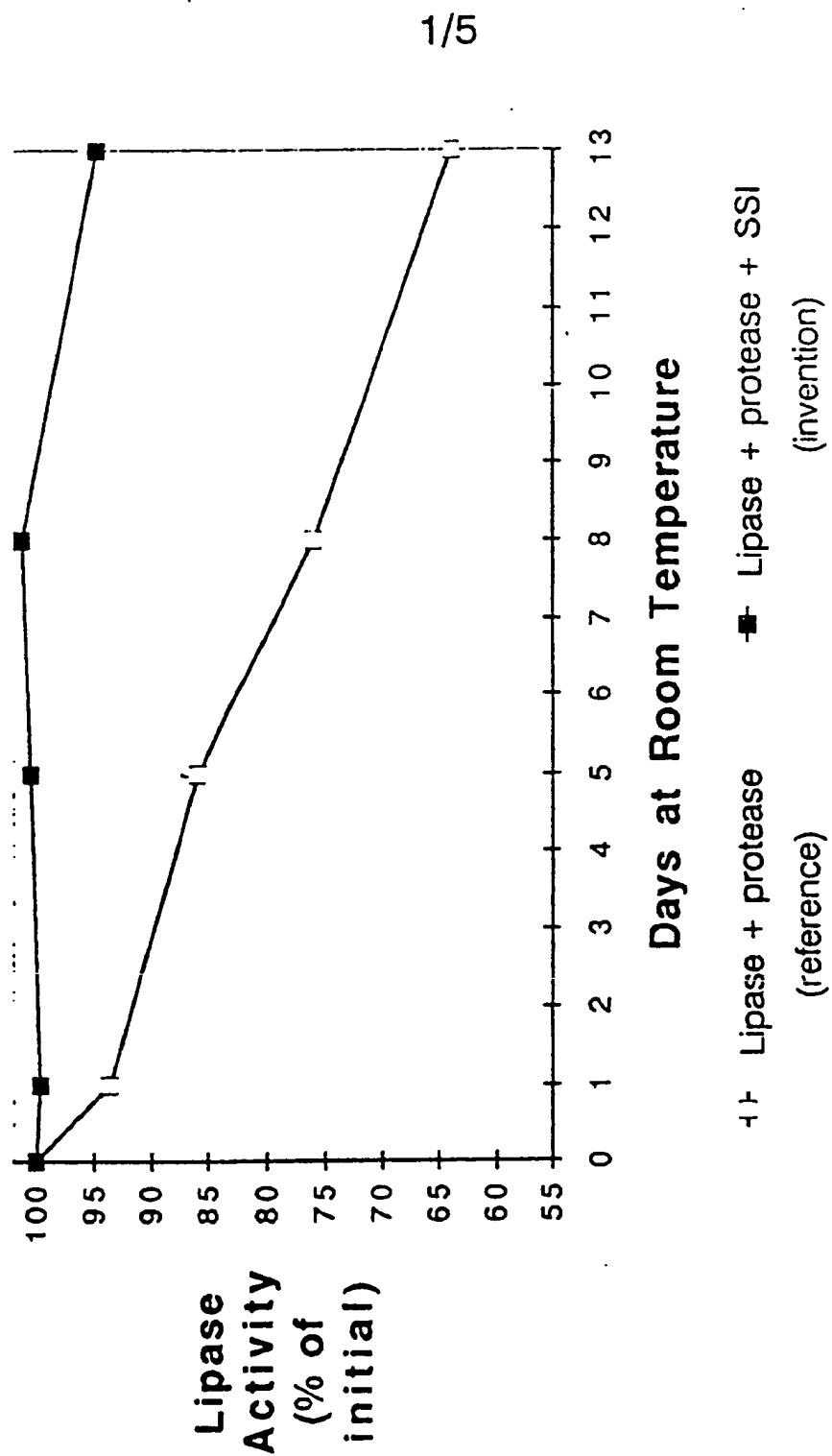


Fig. 1

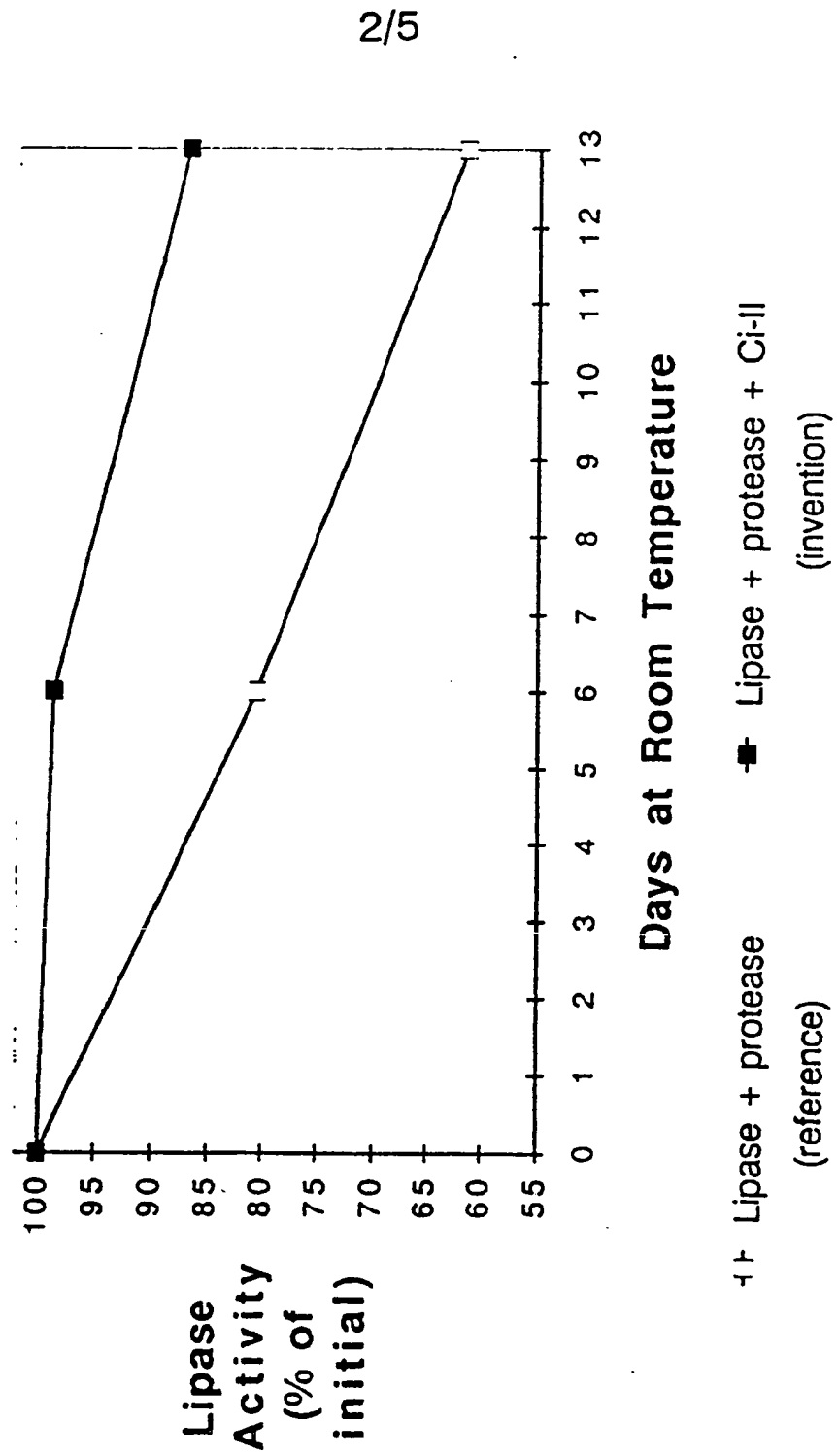


Fig. 2

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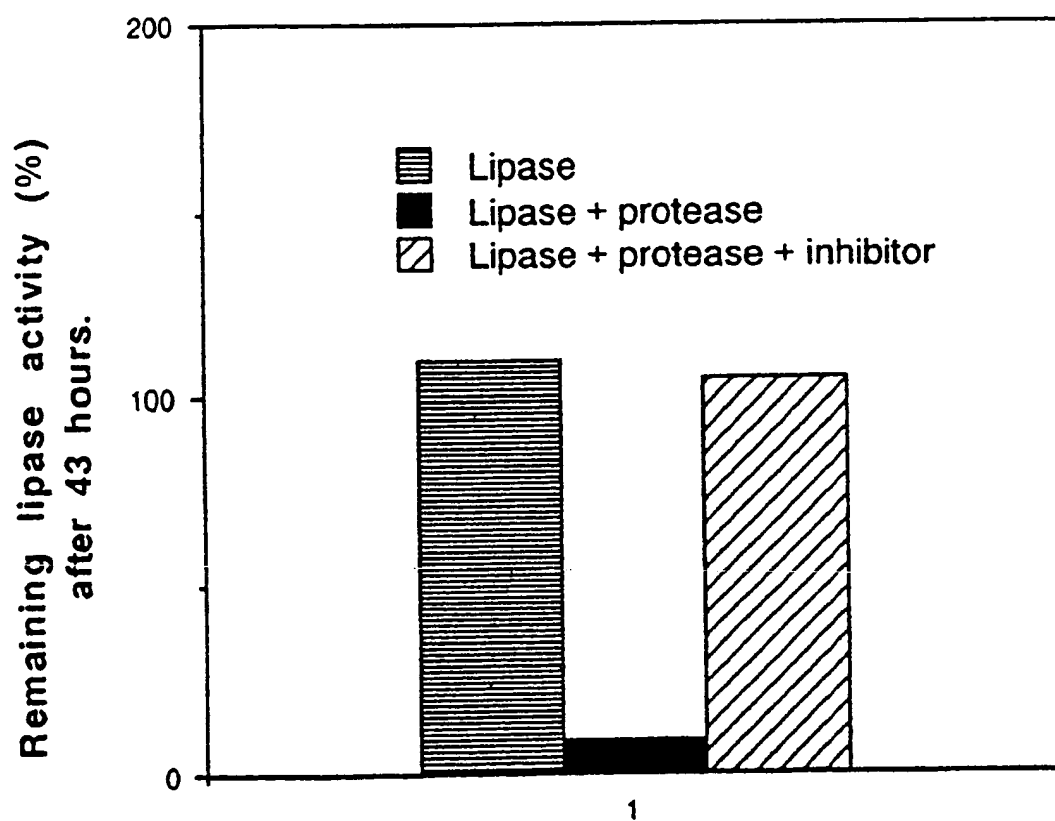


Fig. 3

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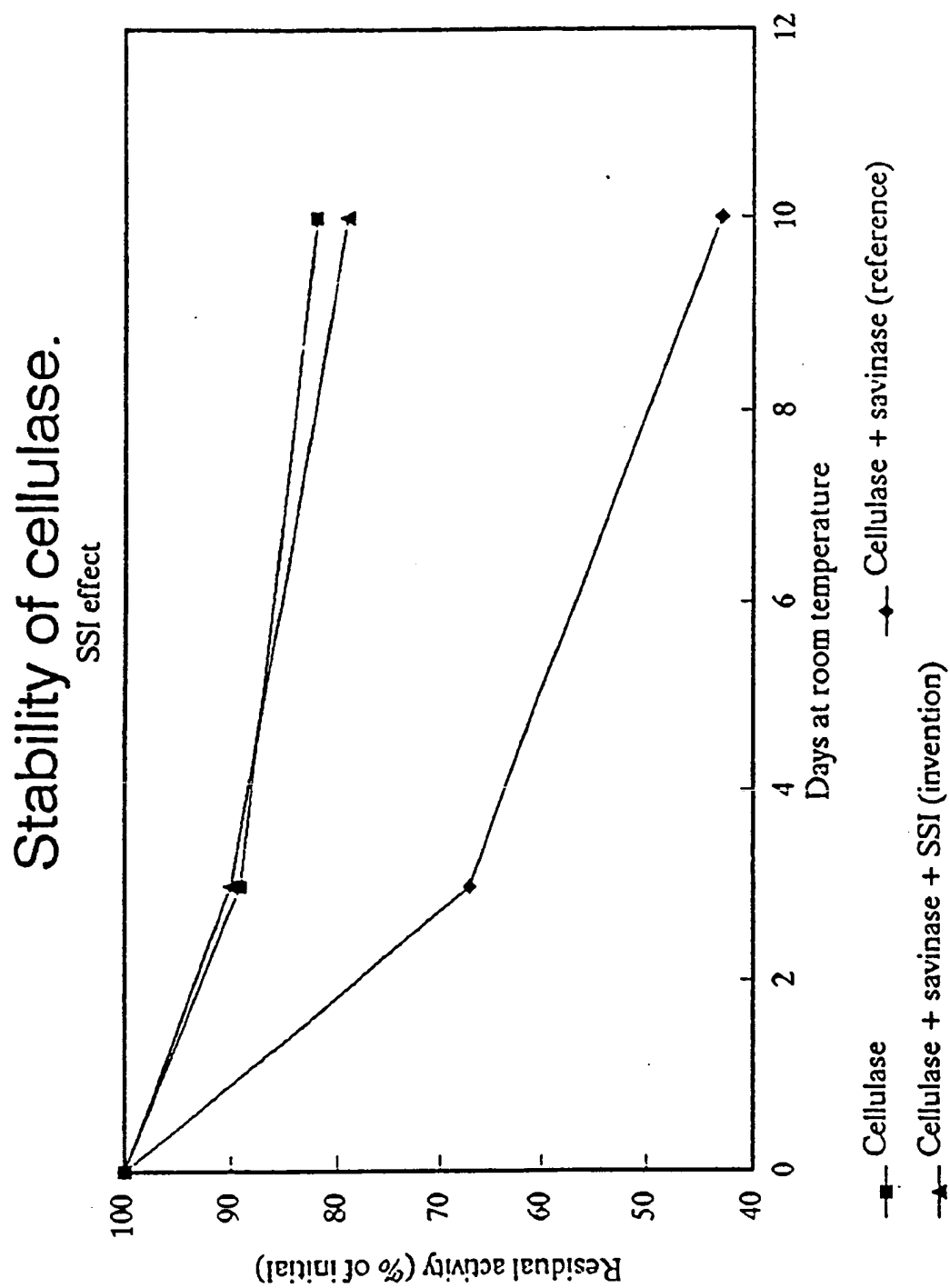


Fig. 4

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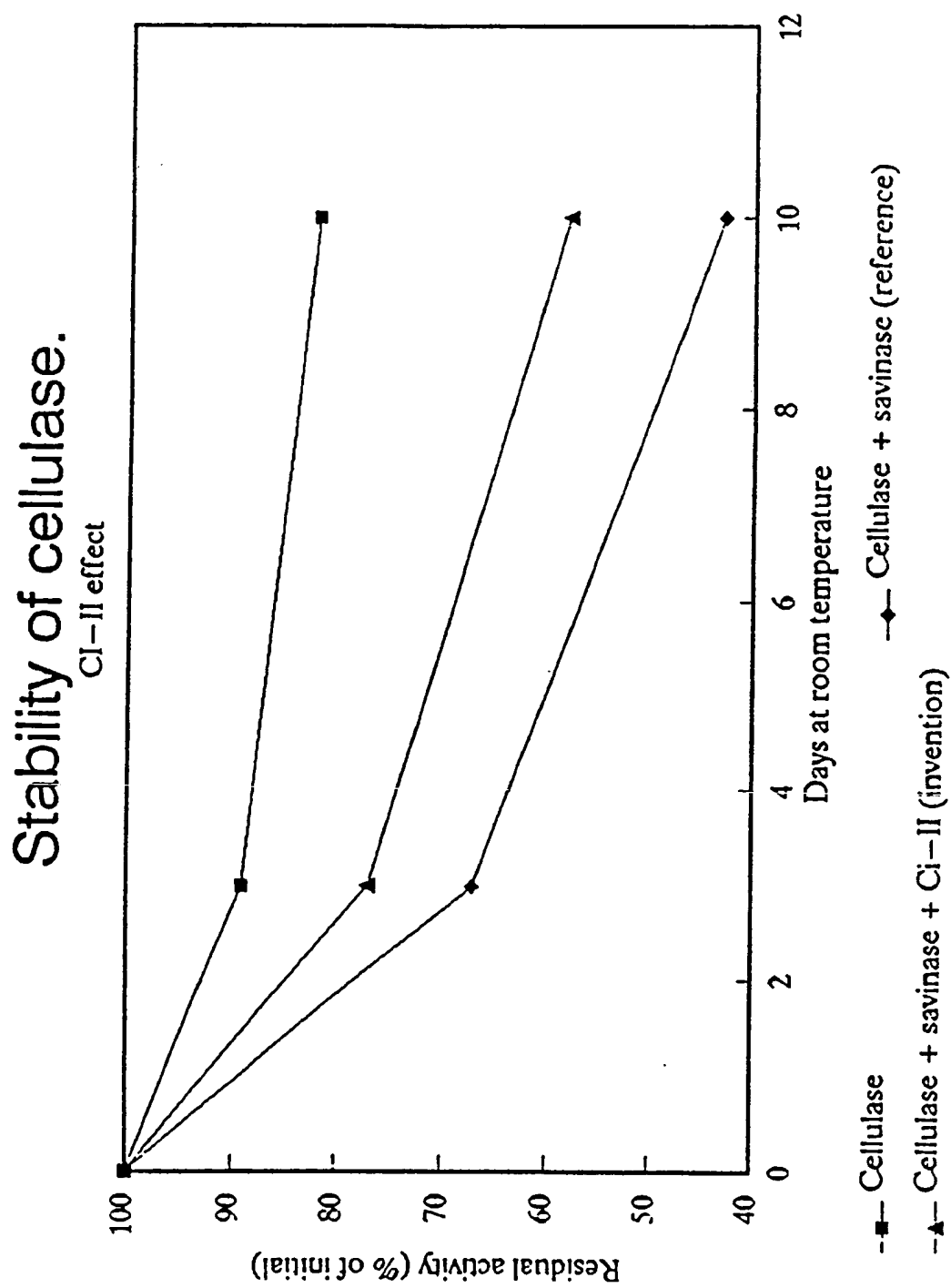
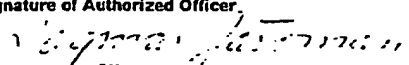
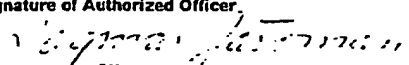
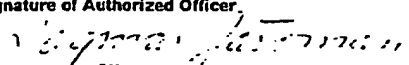


Fig. 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00243

| I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 11 D 3/386 | | | | | | | | | | | | | | |
|--|---|-------------------------------------|--|--|--|---|---|------|---|---|------|---|--|------|
| II. FIELDS SEARCHED <div style="text-align: center;">Minimum Documentation Searched⁷</div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%;">Classification System</th> <th style="width: 75%;">Classification Symbols</th> </tr> <tr> <td style="height: 40px; vertical-align: bottom;">IPC5</td> <td style="vertical-align: bottom;">C 11 D</td> </tr> </table> | | | Classification System | Classification Symbols | IPC5 | C 11 D | | | | | | | | |
| Classification System | Classification Symbols | | | | | | | | | | | | | |
| IPC5 | C 11 D | | | | | | | | | | | | | |
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| III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%;">Category *</th> <th style="width: 60%;">Citation of Document,¹¹ with indication, where appropriate, of the relevant passages¹²</th> <th style="width: 30%;">Relevant to Claim No.¹³</th> </tr> </thead> <tbody> <tr> <td style="vertical-align: top;">P,X</td> <td>US, A, 5039446 (DAVID A. ESTELL) 13 August 1991, see column 7, line 4 - line 12; column 7, line 45 - line 67; abstract; claims 1-8 --</td> <td style="vertical-align: top;">1-16</td> </tr> <tr> <td style="vertical-align: top;">A</td> <td>US, A, 4566985 (BRUNO ET AL) 28 January 1986, see the whole document --</td> <td style="vertical-align: top;">1-16</td> </tr> <tr> <td style="vertical-align: top;">A</td> <td>Patent Abstracts of Japan, Vol 12, No 155, C494, abstract of JP 62-269689, publ 1987-11-24 (SHOWA DENKO K.K.) -- -----</td> <td style="vertical-align: top;">1-16</td> </tr> </tbody> </table> | | | Category * | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ | P,X | US, A, 5039446 (DAVID A. ESTELL) 13 August 1991, see column 7, line 4 - line 12; column 7, line 45 - line 67; abstract; claims 1-8 -- | 1-16 | A | US, A, 4566985 (BRUNO ET AL) 28 January 1986, see the whole document -- | 1-16 | A | Patent Abstracts of Japan, Vol 12, No 155, C494, abstract of JP 62-269689, publ 1987-11-24 (SHOWA DENKO K.K.) -- ----- | 1-16 |
| Category * | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ | | | | | | | | | | | | |
| P,X | US, A, 5039446 (DAVID A. ESTELL) 13 August 1991, see column 7, line 4 - line 12; column 7, line 45 - line 67; abstract; claims 1-8 -- | 1-16 | | | | | | | | | | | | |
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| A | Patent Abstracts of Japan, Vol 12, No 155, C494, abstract of JP 62-269689, publ 1987-11-24 (SHOWA DENKO K.K.) -- ----- | 1-16 | | | | | | | | | | | | |
| <div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div> | | | | | | | | | | | | | | |
| IV. CERTIFICATE <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; padding: 5px;"> Date of the Actual Completion of the International Search 28th November 1991 </td> <td style="width: 50%; padding: 5px;"> Date of Mailing of this International Search Report 1991 -12- 02 </td> </tr> <tr> <td style="width: 50%; padding: 5px;"> International Searching Authority <div style="text-align: center;">SWEDISH PATENT OFFICE</div> </td> <td style="width: 50%; padding: 5px;"> Signature of Authorized Officer <div style="text-align: center;">  Dagmar Järvman </div> </td> </tr> </table> | | | Date of the Actual Completion of the International Search 28th November 1991 | Date of Mailing of this International Search Report 1991 -12- 02 | International Searching Authority <div style="text-align: center;">SWEDISH PATENT OFFICE</div> | Signature of Authorized Officer <div style="text-align: center;">  Dagmar Järvman </div> | | | | | | | | |
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| International Searching Authority <div style="text-align: center;">SWEDISH PATENT OFFICE</div> | Signature of Authorized Officer <div style="text-align: center;">  Dagmar Järvman </div> | | | | | | | | | | | | | |

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 91/00243**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
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| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
| US-A- 5039446 | 91-08-13 | NONE | |
| <hr/> | | | |
| US-A- 4566985 | 86-01-28 | NONE | |

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| (51) International Patent Classification ⁵ : C11D 3/386 | A1 | (11) International Publication Number: WO 92/03529 (43) International Publication Date: 5 March 1992 (05.03.92) |
| (21) International Application Number: PCT/DK91/00243 (22) International Filing Date: 23 August 1991 (23.08.91) (30) Priority data: 2042/90 24 August 1990 (24.08.90) DK (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK). (72) Inventors; and (75) Inventors/Applicants (for US only) : MIKKELSEN, Jan, Møller [DK/DK]; Snerlevej 20, DK-2820 Gentofte (DK). HANSEN, Lone, Kierstein [DK/DK]; Ulrikkenborg Allé 55, DK-2800 Lyngby (DK). (74) Common Representative: NOVO NORDISK A/S; Patent Department, Novo Allé, DK-2880 Bagsvaerd (DK). | | (81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US. Published <i>With international search report.</i> |
| (54) Title: ENZYMATIC DETERGENT COMPOSITION AND METHOD FOR ENZYME STABILIZATION (57) Abstract The invention relates to a detergent composition comprising a protease and one or more other enzymes, as well as comprising a reversible protease inhibitor of the peptide or protein type. | | |

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⁺ Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

ENZYMATIC DETERGENT COMPOSITION AND METHOD FOR ENZYME STABILIZATION

TECHNICAL FIELD

The present invention relates to a detergent composition comprising
5 a protease and a second enzyme (which may be another protease or a non-
proteolytic enzyme), to a method for stabilizing an enzyme in the presence of a
protease and to an enzymatic detergent additive comprising a protease and a
second enzyme.

BACKGROUND ART

10 Proteases are widely used as ingredients in commercial detergents,
including liquids. Two different proteases may be used together (US 4,511,490,
WO 88/03946). Other enzyme types (i.e. non-proteolytic) may also be used in
detergents, e.g. amylase, cellulase, lipase or peroxidase.

A major problem in formulating enzymatic detergents, especially
15 liquid detergents, is that of ensuring enzyme stability during storage. For a
detergent containing a protease together with another enzyme, the stability
problem is aggravated as the protease is liable to digest and deactivate the other
enzyme (i.e. the other protease or the non-proteolytic enzyme).

WO 89/04361 discloses a detergent containing a protease and a
20 lipase, where improved lipase stability is achieved by selecting a specified groups
of lipases and a specified group of proteases.

STATEMENT OF THE INVENTION

We have found that, surprisingly, the stability of an enzyme in a
detergent containing a protease can be improved by incorporation of a reversible
25 protease inhibitor of the peptide or protein type.

Accordingly, the invention provides a detergent composition comprising a protease and one or more other enzymes, characterized by further comprising a reversible protease inhibitor of the peptide or protein type. In another aspect, the invention provides a method for stabilizing an enzyme in the presence of a protease, characterized by incorporating a protease inhibitor. A further aspect of the invention provides an enzymatic detergent additive comprising a protease and one or more other enzymes in the form of a stabilized liquid or a non-dusting granulate, characterized by further comprising a reversible protease inhibitor of the peptide- or protein-type.

10 JP-A 62-269689 discloses improvement of the stability of a protease in a liquid detergent by incorporation of a protease inhibitor, but no other enzymes were present.

DETAILED DESCRIPTION OF THE INVENTION

Protease

15 The protease used in the invention is preferably of microbial origin. It may be a serine protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, e.g. subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (both described in WO 89/06279) and mutant
20 subtilins such as those described in WO 89/06279. Examples of commercial *Bacillus* subtilisins are Alcalase®, Savinase® and Esperase®, products of Novo Nordisk A/S. Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270.

The amount of protease in the detergent will typically be 0.2-40 μM ,
25 especially 1-20 μM (generally 5-1000 mg/l, especially 20-500 mg/l).

Other enzymes

The other enzyme(s) used in the invention may be another protease (e.g. of the type described above) or a non-proteolytic enzyme, e.g. an amylase, a cellulase, a lipase or an oxidoreductase, such as a peroxidase. The non-
5 proteolytic enzyme is preferably of microbial origin, e.g. derived from a strain of *Bacillus*, *Humicola*, *Pseudomonas*, *Coprinus* or *Fusarium*.

The amount of the other enzyme(s) in the detergent will typically be 0.2-40 μ M, especially 1-20 μ M (generally 5-1000 mg/l, especially 20-500 mg/l).

Inhibitor

10 The inhibitor used in the invention may be any inhibitor of the peptide or protein type that reversibly inhibits the protease in question, e.g. those described in Lakowski, Jr. & Kato, *Ann.Rev.Biochem.* (1980) 49:593-626 and S. Murao et al., in *Protein Protease Inhibitor - The Case of Streptomyces subtilisin Inhibitor* (1985) at pp. 1-14. Examples are trypsin inhibitors of Family IV
15 (described in the cited references) and subtilisin inhibitors of family III, VI and VII. More particular examples are *Streptomyces subtilisin inhibitor* (SSI); plasminostreptin from *Streptomyces antifibrinolyticus*; barley subtilisin inhibitor Cl-1 (e.g. described in Williamson et al., *Plant Mol. Biol.* 10, 1988, pp. 521-535) and Cl-2 (e.g. described in Williamson et al., *Eur. J. Biochem.* 165, 1987, pp. 99-106);
20 potato subtilisin inhibitor I (e.g. described in Cleveland et al., *Plant Mol. Biol.* 8, 1988, pp. 199-207); tomato subtilisin inhibitor (e.g. described in Graham et al., *J. Biol. Chem.* 260, 1985, pp. 6555-6560); eglin C from leech (e.g. described in Seemüller et al., *Hoppe-Seylers Z. Physiol. Chem.* 361, 1980, pp. 1841-1846); *Vicia faba* subtilisin inhibitor (e.g. described in Svendsen et al., *Carlsberg Res. Commun.* 49, 1984, pp. 493-502); and leupeptin inhibitor (e.g. described in S. Kondo et al., *J. Antibiot.* 22, 1969, pp. 558-568).

Furthermore, the inhibitor may be a modified subtilisin inhibitor of Family VI with a weaker binding affinity for the protease. Such a modified inhibitor may have one or more of the following amino acid substitutions at the indicated
30 positions (numbered from the reactive site of the inhibitor, P1, P2 etc. are in the

direction of the N-terminal and P'1, P'2 etc. are in the direction of the C-terminal of the inhibitor molecule):

P4: Val, Pro, Trp, Ser, Glu or Arg

P3: Tyr, Glu, Ala, Arg, Pro, Ser, Lys or Trp

5 P2: Ser, Lys, Arg, Pro, Glu, Val, Tyr, Trp or Ala

P1: Arg, Tyr, Pro, Trp, Glu, Val, Ser, Lys or Ala

P'1: Gln, Ser, Thr, Ile or Pro,

P'2: Val, Glu, Arg, Pro or Trp,

P'3: Glu, Gln, Asn, Val, Phe or Tyr.

10 A preferred modified inhibitor is Cl-2 substituted with Arg, Pro or Glu at position P3, Lys or Arg at P2, and/or Glu, Arg or Pro at P1.

Modified inhibitors may be produced by known recombinant DNA techniques. Briefly, a DNA sequence (cDNA or a synthetic gene) encoding a known inhibitor is subjected to mutagenesis in order to replace the codon(s) for
15 the amino acid(s) to be substituted with a new codon (codons) for the desired amino acid substitution(s). This may preferably be carried out by oligonucleotide-directed site-specific mutagenesis in bacteriophage M13 vectors (e.g. M.J. Zoller and M. Smith, Meth. Enzymol. 100 (1983) 468-500), in double-stranded DNA vectors (e.g. Y. Morinaga et al., Biotechnology (July 1984) 636-639), or by the
20 polymerase chain reaction (PCR) (e.g. R. Higuchi, Nucl. Acids. Res. 16 (1988) 7351-7367).

The mutant gene is subsequently expressed in a suitable host strain. Suitable hosts are bacteria (e.g. strains of *Escherichia coli* or *Bacillus*), fungi (e.g. strains of *Saccharomyces cerevisiae* or filamentous fungi like *Aspergillus*), plants
25 such as tomato or potato or established human or animal cell lines. To accomplish expression, the mutant gene has to be inserted in an expression plasmid with promoter and terminator DNA elements for the formation of translatable mutant inhibitor mRNA in vivo. The plasmid is introduced into the host by genetic transformation. The choice of expression plasmid is dependent
30 on the type of host strain used. The expression of the mutant inhibitor may be

done intracellularly or extracellularly. In the latter case, the DNA sequence coding for the mutant inhibitor is fused in frame to a DNA sequence encoding a suitable peptide signalling secretion. The secretion signal should preferably be cleaved off in vivo, resulting in secretion of the mature mutant inhibitor into the growth medium.

The amount of inhibitor preferably corresponds to a molar ratio of inhibitor reactive site to protease active site above 0.6, more preferably above 0.8 and most preferably above 1. The ratio is generally below 10, usually below 5.

The type and amount of inhibitor is preferably chosen so as to provide at least 60% (e.g. at least 80%) inhibition in the detergent as such and below 10% inhibition when the detergent is diluted with water for use in washing, typically at a concentration of 0.3-10 g/l.

Detergent

The detergent of the invention may be in any convenient form, e.g. powder, granules or liquid. The invention is particularly applicable to the formulation of liquid detergents where enzyme stability problems are pronounced. A liquid detergent may be aqueous, typically containing 20-70% water and 0-20% organic solvent (hereinafter, percentages by weight).

The detergent comprises surfactant which may be anionic, non-ionic, cationic, amphoteric or a mixture of these types. The detergent will usually contain 5-30% anionic surfactant such as linear alkyl benzene sulphonate (LAS), alpha-olefin sulphonate (AOS), alcohol ethoxy sulphate (AES) or soap. It may also contain 3-20% anionic surfactant such as nonyl phenol ethoxylate or alcohol ethoxylate.

The pH (measured in aqueous detergent solution) will usually be neutral or alkaline, e.g. 7-10. The detergent may contain 1-40% of a detergent builder such as zeolite, phosphate, phosphonate, citrate, NTA, EDTA or DTPA, or it may be unbuilt (i.e. essentially free of a detergent builder). It may also contain other conventional detergent ingredients, e.g. fabric conditioners, foam boosters, bactericides, optical brighteners and perfumes.

Detergent additive

The protease, other enzyme(s) and inhibitor may be included in the detergent of the invention by separate addition or by adding the combined additive provided by the invention. The additive will usually contain 0.2-8 mM
5 protease (0.5-20%) and 0.2-8 mM (0.5-20%) of the second enzyme, and have an inhibitor/protease ratio as described above.

The detergent additive may be in liquid form for incorporation in a liquid detergent. A liquid additive may contain 20-90% propylene glycol; 0.5-3% (as Ca) of a soluble calcium salt; 0-10% glycerol; minor amounts of short-chain
10 fatty acids and carbohydrate; and water up to 100%.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is illustrated in further detail in the following examples with reference to the appended drawings, wherein

Fig. 1 is a graph showing the residual activity (in %) after 13 days at
15 room temperature of lipase in a detergent composition containing lipase and protease alone compared to a composition containing lipase, protease and Streptomyces subtilisin inhibitor;

Fig. 2 is a graph showing the residual activity (in %) after 13 days at room temperature of lipase in a detergent composition containing lipase and
20 protease alone compared to a composition containing lipase, protease and barley subtilisin inhibitor CI-2;

Fig. 3 is a graph showing the residual activity (in %) after 43 hours at room temperature of lipase in the presence of protease with or without added leupeptin inhibitor; and

Fig. 4 is a graph showing the residual activity (in %) after 10 days at room temperature of cellulase in the presence of protease with or without added Streptomyces subtilisin inhibitor; and

Fig. 5 is a graph showing the residual activity (in %) after 10 days at room temperature of cellulase in the presence of protease with or without added CI-2 inhibitor.

EXAMPLE 1

A concentrated liquid detergent was formulated as follows (% by weight of active substance):

| | | | |
|----|------------------------------|-------|-------|
| 10 | LAS (Nansa 1169/p) | 5% | |
| | AES (Berol 452) | 5 | |
| | Oleic:coco fatty acid (1:1) | 10 | |
| | AE (Dobanol 25-7) | 15 | |
| | Triethanolamine | 5 | |
| 15 | NaOH | | 1.1 |
| | SXS | 3 | |
| | Ethanol | 4.8 | |
| | Propylene glycol | 8 | |
| | Glycerol | 2 | |
| 20 | CaCl ₂ | | 0.045 |
| | Sodium citrate | 0.089 | |
| | Phosphonate (Dequest 2060 S) | 0.5 | |
| | pH | 8.0 | |

A detergent according to the invention was prepared by addition of *Streptomyces* subtilisin inhibitor (SSI, 0.05 mg/ml, 4.5 μ M) to a detergent of the composition: 52 (v/v) % of the above concentrated detergent in water containing

10 mg/ml (300 μ M) *Humicola* lipase (LipolaseTM) and 0.1 mg/ml (3.6 μ M) Savinase .

Another detergent was prepared by addition of inhibitor CI-2 (0.03 mg/ml, 3.3 μ M) to a detergent of the composition 55 (v/v) % concentrated 5 detergent in water containing 10 mg/ml (300 μ M) *Humicola* lipase (LipolaseTM) and 0.1 mg/ml (3.6 μ M) Savinase®.

Both detergents were stored for 13 days at room temperature together with reference detergents of the same composition, but without inhibitor. The lipase activity was measured at various times and expressed in % of initial 10 lipase activity. The results, shown in the two figures, demonstrate a pronounced stabilizing effect on the lipase by addition of the protease inhibitor.

EXAMPLE 2

The protection of lipase from proteolytic degradation in the presence of a protease inhibitor was determined by adding 0.67 g/l leupeptin inhibitor to a 15 mixture of 0.5 g/l *Pseudomonas cepacia* lipase and 2 g/l *Fusarium* protease in 50 mM Tris-HCl, pH 8.0, at 20°C and measuring the residual lipase activity (in %) after 43 hours. From the results shown in Fig. 3 it appears that there is very little degradation of the lipase in the presence of the leupeptin inhibitor, whereas the lipase is almost completely degraded when no inhibitor is added. The protease 20 activity may be restored by dilution. After storage for 43 hours followed by 100-fold dilution, the protease activity was 327 U/ml (U = arbitrary units established by means of the synthetic substrate N-p-tosyl-Gly-Pro-Arg-p-nitroanilide) in the preparation containing lipase and protease, and 366 U/ml in the equivalent preparation which also contains the leupeptin inhibitor.

EXAMPLE 3

A concentrated liquid detergent was formulated as follows (% by weight of active substance):

| | | |
|---|--------------------|-----|
| | LAS (Nansa 1169 P) | 10% |
| 5 | AEO (Berol 160) | 15% |
| | Ethanol | 10% |
| | Triethanolamine | 5% |

A detergent according to the invention was prepared by addition of *Streptomyces subtilisin* inhibitor (SSI, 0.09 mg/ml, 7.7 μ M) to a detergent (90% (w/w) of the above concentrated detergent in water) containing 0.12 mg/ml (3.3 μ M) *Humicola* cellulase and 0.18 mg/ml (6.7 μ M) Savinase®.

Another detergent was prepared by addition of inhibitor CI-2 (0.07 mg/ml, 7.8 μ M) to a detergent (90% (w/w) of the above concentrated detergent in water) containing 0.12 mg/ml (3.3 μ M) *Humicola* cellulase and 0.18 mg/ml (6.7 μ M) Savinase®.

Both detergents were stored for 10 days at room temperature together with a reference detergent without any inhibitor. The residual cellulase activity was measured at various times and expressed in % of initial cellulase activity. The results, shown in Fig. 4 and 5 demonstrate a pronounced stabilizing effect on the cellulase by addition of protease inhibitor, especially with SSI.

CLAIMS

1. A detergent composition comprising a protease and one or more other enzymes, characterized by further comprising a reversible protease inhibitor of the peptide or protein type.
- 5 2. A composition according to the preceding claim, wherein the molar ratio of inhibitor reactive site to protease active site is above 0.6, preferably 1-10.
3. A composition according to either preceding claim, wherein the amount of protease is 0.2-40 μM , preferably 1-20 μM .
4. A composition according to Claim 1, wherein the protease is a
10 serine protease, preferably an alkaline microbial protease or a trypsin-like protease.
5. A composition according to Claim 4, wherein the alkaline microbial protease is a subtilisin, preferably derived from *Bacillus*, most preferably subtilisin Novo, subtilisin Carlsberg, BPN', subtilisin 309, subtilisin 147 or subtilisin 168.
- 15 6. A composition according to Claim 4, wherein the trypsin-like protease is trypsin or is derived from *Fusarium*.
7. A composition according to any preceding claim, wherein the inhibitor is a trypsin inhibitor of family IV or a subtilisin inhibitor of family III, VI or VII.
- 20 8. A composition according to any preceding claim, wherein the other enzyme is also a protease, preferably of the type defined in any of Claims 4 - 6.

9. A composition according to any of Claims 1 - 7, wherein the other enzyme is a non-proteolytic enzyme, preferably an amylase, a cellulase, a lipase or an oxidoreductase, such as a peroxidase.
10. A composition according to the preceding claim, wherein the
5 enzyme is of microbial origin, preferably derived from *Bacillus*, *Humicola*, *Pseudomonas*, *Coprinus* or *Fusarium*.
11. A composition according to any preceding claim, wherein the degree of protease inhibition in the detergent is at least 60%.
12. A composition according to any preceding claim, wherein the
10 degree of protease inhibition in a 1% detergent solution in water is below 10%.
13. An aqueous liquid detergent composition according to any preceding claim.
14. A method for stabilizing an enzyme in the presence of a protease, characterized by incorporating a protease inhibitor of the peptide or protein type.
- 15 15. A method according to Claim 14 for stabilizing an enzyme in a detergent, preferably an aqueous liquid detergent.
16. An enzymatic detergent additive comprising a protease and one or more other enzymes in the form of a stabilized liquid or a non-dusting granulate, characterized by further comprising a reversible protease inhibitor of the peptide
20 or protein type.

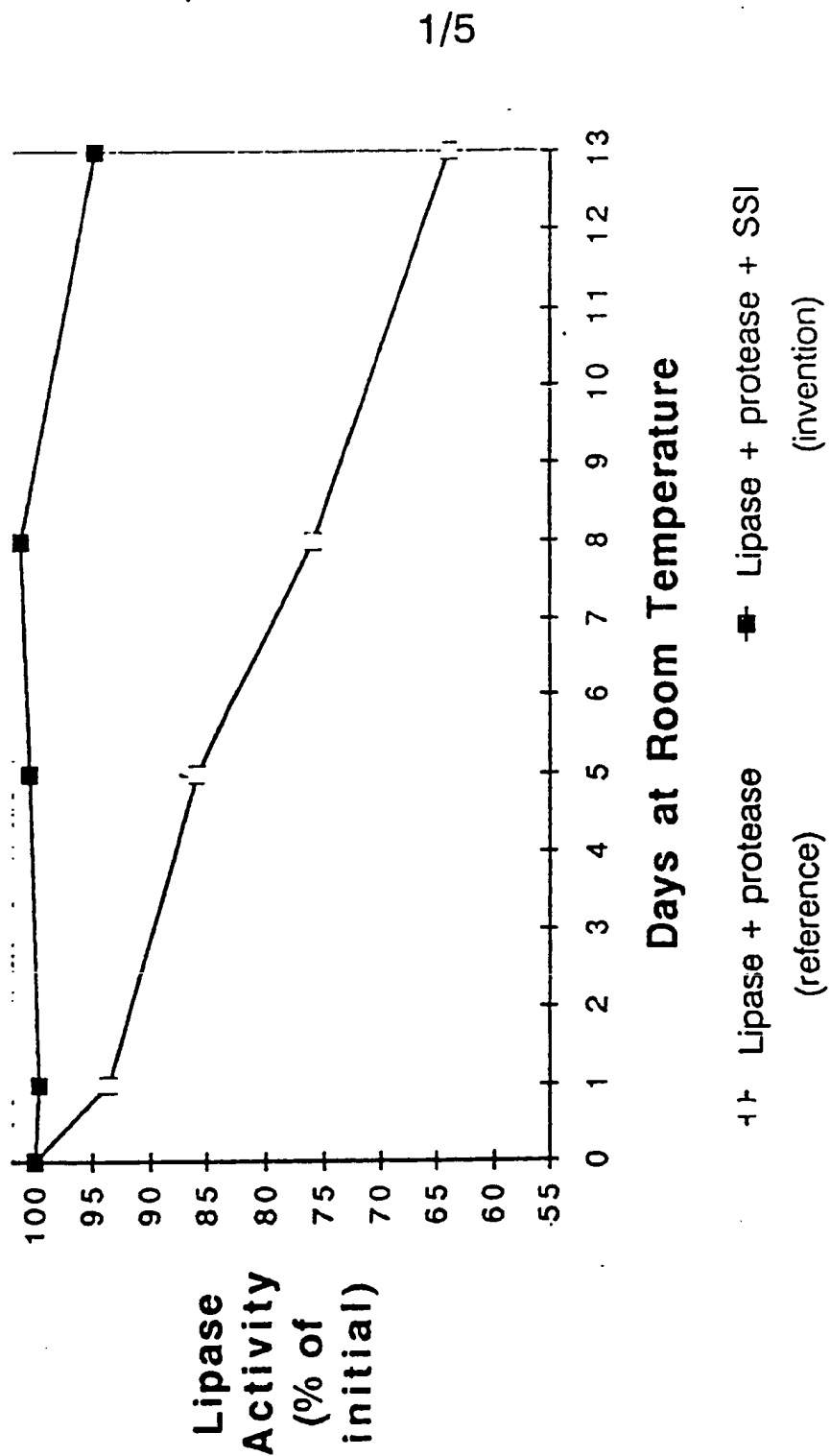


Fig. 1

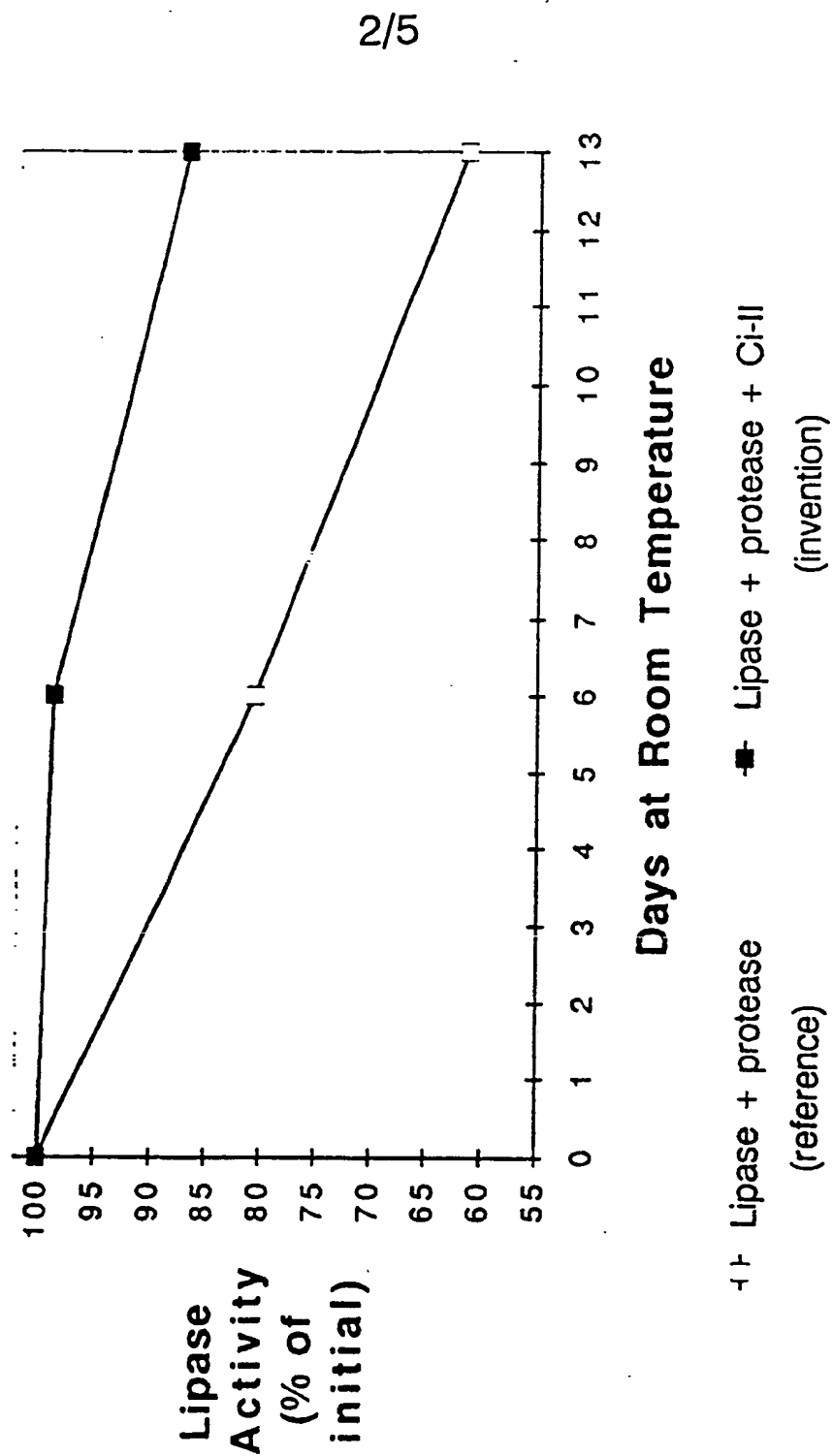


Fig. 2

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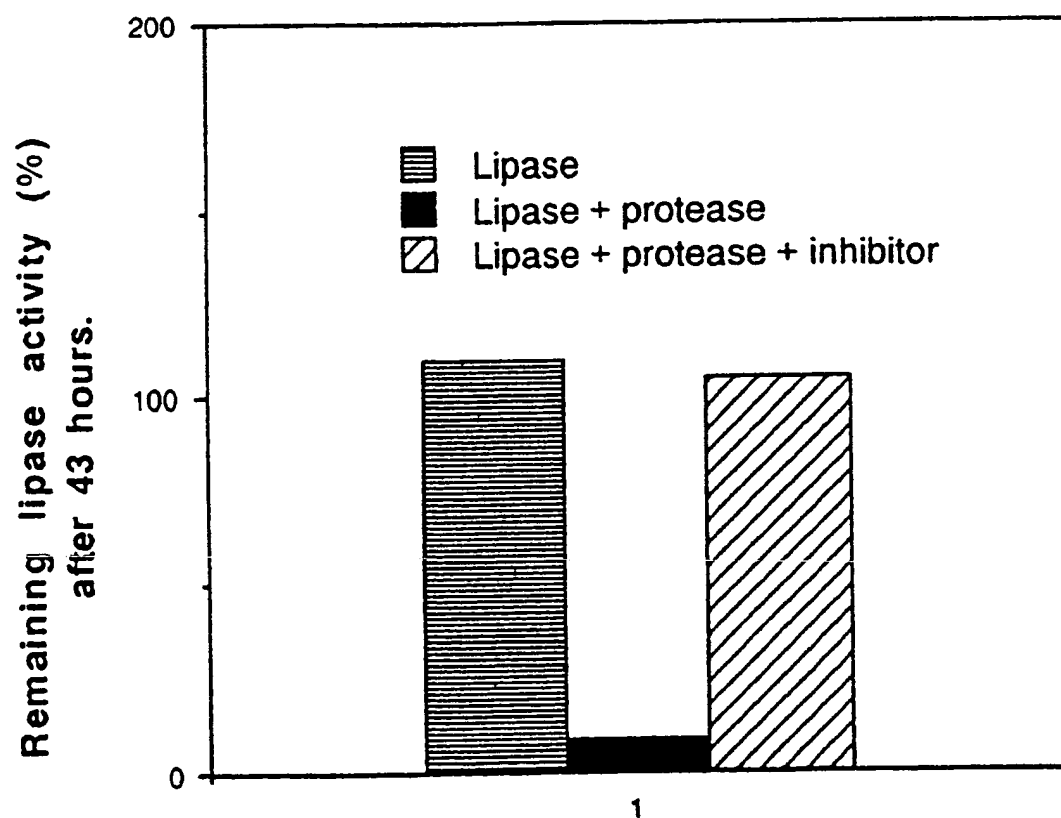


Fig. 3

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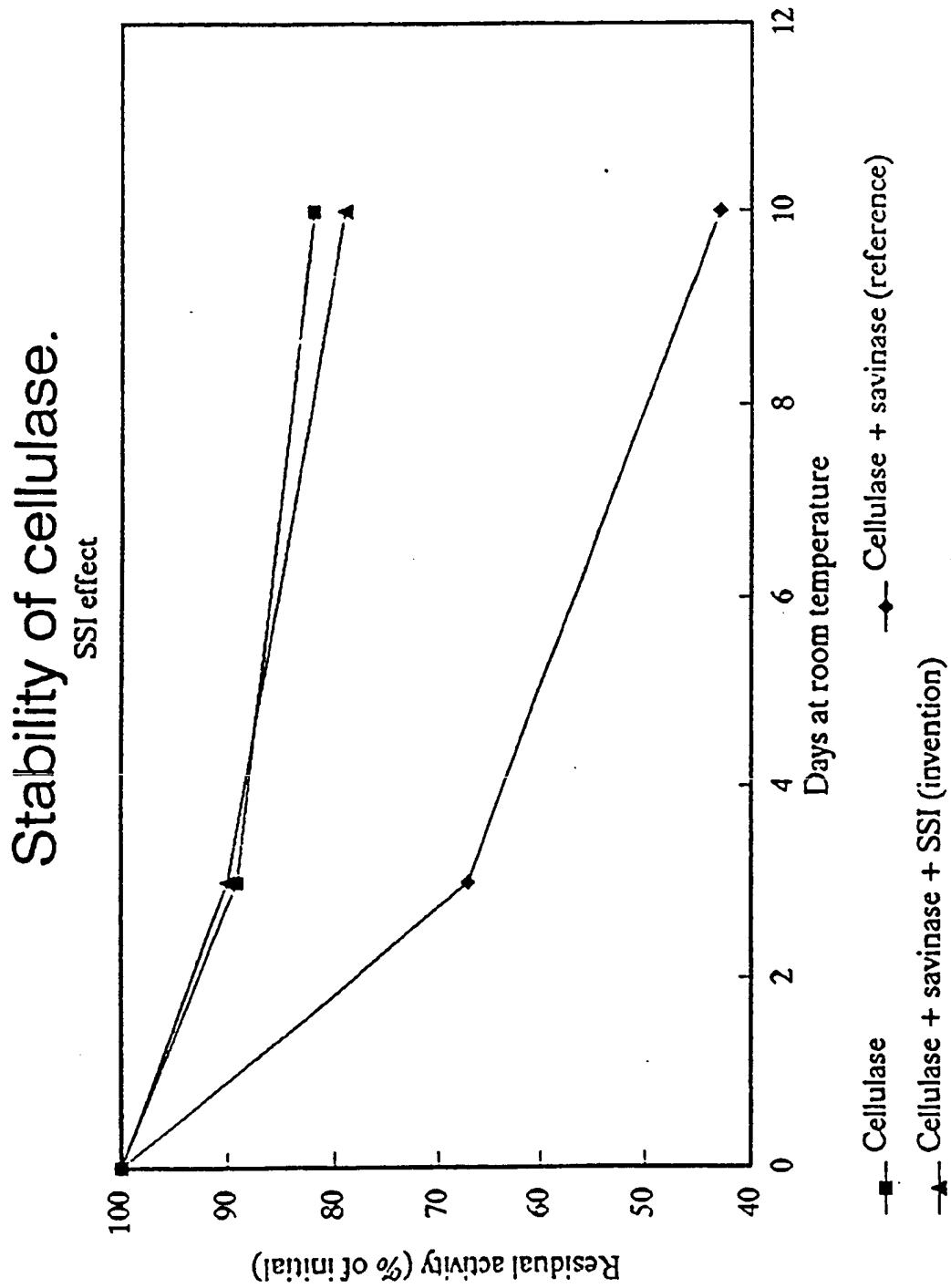


Fig. 4

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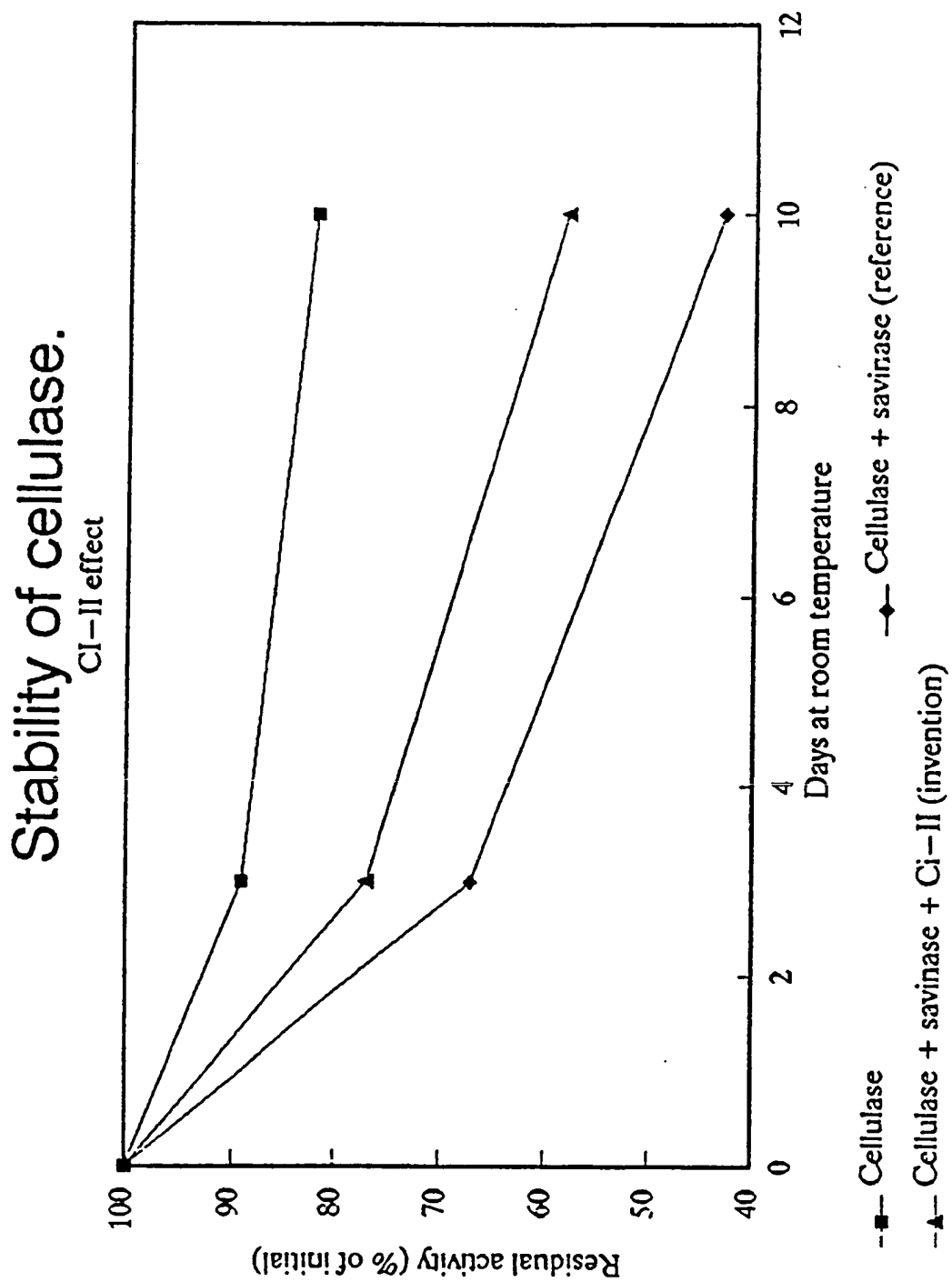
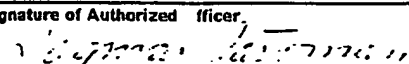
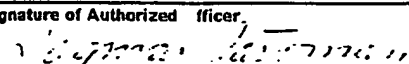
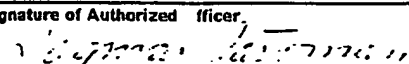


Fig. 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00243

| I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 11 D 3/386 | | | | | | | | | | | | | | |
|--|---|-------------------------------------|---|--|-------------------------------------|---------------------|---|---------------------------------|------------------------------|---|------|---|--|------|
| II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 25%; border-bottom: 1px solid black; padding: 2px;">Classification System</td> <td style="border-bottom: 1px solid black; padding: 2px;">Classification Symbols</td> </tr> <tr> <td style="padding: 5px;">IPC5</td> <td style="padding: 5px;">C 11 D</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched⁸</div> <p style="padding: 5px;">SE,DK,FI,NO classes as above</p> | | | Classification System | Classification Symbols | IPC5 | C 11 D | | | | | | | | |
| Classification System | Classification Symbols | | | | | | | | | | | | | |
| IPC5 | C 11 D | | | | | | | | | | | | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black; padding: 2px;">Category *</th> <th style="width: 60%; border-bottom: 1px solid black; padding: 2px;">Citation of Document,¹¹ with indication, where appropriate, of the relevant passages¹²</th> <th style="width: 30%; border-bottom: 1px solid black; padding: 2px;">Relevant to Claim No.¹³</th> </tr> <tr> <td style="vertical-align: top; padding: 5px;">P,X</td> <td style="padding: 5px;">US, A, 5039446 (DAVID A. ESTELL) 13 August 1991, see column 7, line 4 - line 12; column 7, line 45 - line 67; abstract; claims 1-8 --</td> <td style="vertical-align: top; padding: 5px;">1-16</td> </tr> <tr> <td style="vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">US, A, 4566985 (BRUNO ET AL) 28 January 1986, see the whole document --</td> <td style="vertical-align: top; padding: 5px;">1-16</td> </tr> <tr> <td style="vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">Patent Abstracts of Japan, Vol 12, No 155, C494, abstract of JP 62-269689, publ 1987-11-24 (SHOWA DENKO K.K.) -- -----</td> <td style="vertical-align: top; padding: 5px;">1-16</td> </tr> </table> | | | Category * | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ | P,X | US, A, 5039446 (DAVID A. ESTELL) 13 August 1991, see column 7, line 4 - line 12; column 7, line 45 - line 67; abstract; claims 1-8 -- | 1-16 | A | US, A, 4566985 (BRUNO ET AL) 28 January 1986, see the whole document -- | 1-16 | A | Patent Abstracts of Japan, Vol 12, No 155, C494, abstract of JP 62-269689, publ 1987-11-24 (SHOWA DENKO K.K.) -- ----- | 1-16 |
| Category * | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ | | | | | | | | | | | | |
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| <div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p>* Special categories of cited documents:¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div> | | | | | | | | | | | | | | |
| IV. CERTIFICATE <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 2px;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black; padding: 2px;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="padding: 5px;">28th November 1991</td> <td style="padding: 5px;">1991 -12- 02</td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 2px;">International Searching Authority</td> <td style="border-bottom: 1px solid black; padding: 2px;">Signature of Authorized Officer</td> </tr> <tr> <td style="padding: 5px; text-align: center;">SWEDISH PATENT OFFICE</td> <td style="padding: 5px; text-align: center;">  Dagmar Järvman </td> </tr> </table> | | | Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report | 28th November 1991 | 1991 -12- 02 | International Searching Authority | Signature of Authorized Officer | SWEDISH PATENT OFFICE |  Dagmar Järvman | | | | |
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**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/DK 91/00243**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 91-10-31. The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
| US-A- 5039446 | 91-08-13 | NONE | |
| US-A- 4566985 | 86-01-28 | NONE | |

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| (51) International Patent Classification ⁵ : C11D 3/386 | A1 | (11) International Publication Number: WO 92/03529 (43) International Publication Date: 5 March 1992 (05.03.92) |
| (21) International Application Number: PCT/DK91/00243 (22) International Filing Date: 23 August 1991 (23.08.91) (30) Priority data: 2042/90 24 August 1990 (24.08.90) DK (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK). (72) Inventors; and (75) Inventors/Applicants (for US only) : MIKKELSEN, Jan, Møller [DK/DK]; Snerlevej 20, DK-2820 Gentofte (DK). HANSEN, Lone, Kierstein [DK/DK]; Ulrikkenborg Allé 55, DK-2800 Lyngby (DK). (74) Common Representative: NOVO NORDISK A/S; Patent Department, Novo Allé, DK-2880 Bagsvaerd (DK). | | (81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US. Published <i>With international search report.</i> |
| (54) Title: ENZYMATIC DETERGENT COMPOSITION AND METHOD FOR ENZYME STABILIZATION (57) Abstract The invention relates to a detergent composition comprising a protease and one or more other enzymes, as well as comprising a reversible protease inhibitor of the peptide or protein type. | | |

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⁺ Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

ENZYMATIC DETERGENT COMPOSITION AND METHOD FOR ENZYME STABILIZATION

TECHNICAL FIELD

The present invention relates to a detergent composition comprising
5 a protease and a second enzyme (which may be another protease or a non-
proteolytic enzyme), to a method for stabilizing an enzyme in the presence of a
protease and to an enzymatic detergent additive comprising a protease and a
second enzyme.

BACKGROUND ART

10 Proteases are widely used as ingredients in commercial detergents,
including liquids. Two different proteases may be used together (US 4,511,490,
WO 88/03946). Other enzyme types (i.e. non-proteolytic) may also be used in
detergents, e.g. amylase, cellulase, lipase or peroxidase.

A major problem in formulating enzymatic detergents, especially
15 liquid detergents, is that of ensuring enzyme stability during storage. For a
detergent containing a protease together with another enzyme, the stability
problem is aggravated as the protease is liable to digest and deactivate the other
enzyme (i.e. the other protease or the non-proteolytic enzyme).

WO 89/04361 discloses a detergent containing a protease and a
20 lipase, where improved lipase stability is achieved by selecting a specified groups
of lipases and a specified group of proteases.

STATEMENT OF THE INVENTION

We have found that, surprisingly, the stability of an enzyme in a
detergent containing a protease can be improved by incorporation of a reversible
25 protease inhibitor of the peptide or protein type.

Accordingly, the invention provides a detergent composition comprising a protease and one or more other enzymes, characterized by further comprising a reversible protease inhibitor of the peptide or protein type. In another aspect, the invention provides a method for stabilizing an enzyme in the presence of a protease, characterized by incorporating a protease inhibitor. A further aspect of the invention provides an enzymatic detergent additive comprising a protease and one or more other enzymes in the form of a stabilized liquid or a non-dusting granulate, characterized by further comprising a reversible protease inhibitor of the peptide- or protein-type.

10 JP-A 62-269689 discloses improvement of the stability of a protease in a liquid detergent by incorporation of a protease inhibitor, but no other enzymes were present.

DETAILED DESCRIPTION OF THE INVENTION

Protease

15 The protease used in the invention is preferably of microbial origin. It may be a serine protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, e.g. subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (both described in WO 89/06279) and mutant
20 subtilins such as those described in WO 89/06279. Examples of commercial *Bacillus* subtilisins are Alcalase®, Savinase® and Esperase®, products of Novo Nordisk A/S. Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270.

The amount of protease in the detergent will typically be 0.2-40 μ M,
25 especially 1-20 μ M (generally 5-1000 mg/l, especially 20-500 mg/l).

Other enzymes

The other enzyme(s) used in the invention may be another protease (e.g. of the type described above) or a non-proteolytic enzyme, e.g. an amylase, a cellulase, a lipase or an oxidoreductase, such as a peroxidase. The non-
5 proteolytic enzyme is preferably of microbial origin, e.g. derived from a strain of *Bacillus*, *Humicola*, *Pseudomonas*, *Coprinus* or *Fusarium*.

The amount of the other enzyme(s) in the detergent will typically be 0.2-40 μ M, especially 1-20 μ M (generally 5-1000 mg/l, especially 20-500 mg/l).

Inhibitor

10 The inhibitor used in the invention may be any inhibitor of the peptide or protein type that reversibly inhibits the protease in question, e.g. those described in Lakowski, Jr. & Kato, *Ann.Rev.Biochem.* (1980) 49:593-626 and S. Murao et al., in *Protein Protease Inhibitor - The Case of Streptomyces subtilisin Inhibitor* (1985) at pp. 1-14. Examples are trypsin inhibitors of Family IV
15 (described in the cited references) and subtilisin inhibitors of family III, VI and VII. More particular examples are *Streptomyces* subtilisin inhibitor (SSI); plasminostreptin from *Streptomyces antifibrinolyticus*; barley subtilisin inhibitor CI-1 (e.g. described in Williamson et al., *Plant Mol. Biol.* 10, 1988, pp. 521-535) and CI-2 (e.g. described in Williamson et al., *Eur. J. Biochem.* 165, 1987, pp. 99-106);
20 potato subtilisin inhibitor I (e.g. described in Cleveland et al., *Plant Mol. Biol.* 8, 1988, pp. 199-207); tomato subtilisin inhibitor (e.g. described in Graham et al., *J. Biol. Chem.* 260, 1985, pp. 6555-6560); eglin C from leech (e.g. described in Seemüller et al., *Hoppe-Seylers Z. Physiol. Chem.* 361, 1980, pp. 1841-1846); *Vicia faba* subtilisin inhibitor (e.g. described in Svendsen et al., *Carlsberg Res.*
25 *Commun.* 49, 1984, pp. 493-502); and leupeptin inhibitor (e.g. described in S. Kondo et al., *J. Antibiot.* 22, 1969, pp. 558-568).

Furthermore, the inhibitor may be a modified subtilisin inhibitor of Family VI with a weaker binding affinity for the protease. Such a modified inhibitor may have one or more of the following amino acid substitutions at the indicated
30 positions (numbered from the reactive site of the inhibitor, P1, P2 etc. are in the

direction of the N-terminal and P'1, P'2 etc. are in the direction of the C-terminal of the inhibitor molecule):

P4: Val, Pro, Trp, Ser, Glu or Arg

P3: Tyr, Glu, Ala, Arg, Pro, Ser, Lys or Trp

5 P2: Ser, Lys, Arg, Pro, Glu, Val, Tyr, Trp or Ala

P1: Arg, Tyr, Pro, Trp, Glu, Val, Ser, Lys or Ala

P'1: Gln, Ser, Thr, Ile or Pro,

P'2: Val, Glu, Arg, Pro or Trp,

P'3: Glu, Gln, Asn, Val, Phe or Tyr.

10 A preferred modified inhibitor is Cl-2 substituted with Arg, Pro or Glu at position P3, Lys or Arg at P2, and/or Glu, Arg or Pro at P1.

Modified inhibitors may be produced by known recombinant DNA techniques. Briefly, a DNA sequence (cDNA or a synthetic gene) encoding a known inhibitor is subjected to mutagenesis in order to replace the codon(s) for
15 the amino acid(s) to be substituted with a new codon (codons) for the desired amino acid substitution(s). This may preferably be carried out by oligonucleotide-directed site-specific mutagenesis in bacteriophage M13 vectors (e.g. M.J. Zoller and M. Smith, Meth. Enzymol. 100 (1983) 468-500), in double-stranded DNA vectors (e.g. Y. Morinaga et al., Biotechnology (July 1984) 636-639), or by the
20 polymerase chain reaction (PCR) (e.g. R. Higuchi, Nucl. Acids. Res. 16 (1988) 7351-7367).

The mutant gene is subsequently expressed in a suitable host strain. Suitable hosts are bacteria (e.g. strains of *Escherichia coli* or *Bacillus*), fungi (e.g. strains of *Saccharomyces cerevisiae* or filamentous fungi like *Aspergillus*), plants
25 such as tomato or potato or established human or animal cell lines. To accomplish expression, the mutant gene has to be inserted in an expression plasmid with promoter and terminator DNA elements for the formation of translatable mutant inhibitor mRNA in vivo. The plasmid is introduced into the host by genetic transformation. The choice of expression plasmid is dependent
30 on the type of host strain used. The expression of the mutant inhibitor may be